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Rapid and direct quantification of viable *Candida* species in whole blood using immunomagnetic separation and solid-phase cytometry

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ABSTRACT

Candida species are a common source of nosocomial bloodstream infections in critically ill patients. The sensitivity of the traditional diagnostic procedure based on blood culture is variable and it usually takes two to four days before growth of *Candida* species is detected. We developed a four-hour method for the quantification of *Candida* species in blood, combining immunomagnetic separation (IMS) with solid-phase cytometry (SPC) using viability labelling. Additionally, *Candida albicans* cells could be identified in real-time by using fluorescent in situ hybridisation. When analysing spiked blood samples, our method was shown to be sensitive and specific with a low detection limit (1 cell/ml of blood). In a proof of concept study, we applied the IMS/SPC method to 16 clinical samples and compared it to traditional blood culture. Our method proved more sensitive than culture (seven samples were positive with IMS/SPC but negative with blood culture) and identification results were in agreement. The IMS/SPC data also suggest that mixed infections might occur frequently as in five samples *C. albicans* and at least one other *Candida* species was found. Additionally, in two cases, high numbers of cells (175-480 cells/ml of blood) were associated with an endovascular source of infection.

Key words: Candidemia, solid-phase cytometry, immunomagnetic separation, rapid quantification

INTRODUCTION

Candida species are a common source of nosocomial bloodstream infections in critically ill patients, with mortality rates exceeding 40%. Rapid detection and identification of *Candida albicans* and other *Candida* species could result in early initiation of adequate antifungal therapy, an important factor reducing morbidity and mortality [3, 16].

At present, the gold standard for the detection of *Candida* species in the bloodstream is culture of blood samples. Although blood culture systems have evolved in recent years from manual to fully automated systems, the diagnostic sensitivity is still variable and differs greatly among studies, with 40 to 82% of blood culture bottles spiked with *Candida* or from patients with proven candidemia showing positive results [4, 6, 9, 10, 18]. Possible explanations are the low numbers of *Candida* cells present in the blood during fungemia (10 - 25 cells per 10 ml of blood) [4, 10, 18], the use of growth media which are not optimal for fungal growth and the presence of antimycotics in the blood [6]. Additionally, it usually takes two to four days before growth of *Candida* species is detected in blood culture bottles [4, 10].

Several studies have shown good results when using PCR on DNA isolated from whole blood for the detection of candidemia [5, 13, 21, 22]. However, one of the problems using PCR is the possibility of detecting DNA from dead and/or degrading yeast cells instead of living yeasts, leading to false-positive results [5, 13, 21, 22]. Also, in many cases only small sample volumes can be used, or a long and cumbersome sample preparation is needed to reduce the influence of inhibitors present in blood [5, 13].

Another approach is to recover yeast cells by immunomagnetic separation (IMS) prior to further analysis. Magnetic beads coated with antibodies are used to

capture the yeast cells present in the clinical sample and separation occurs in a magnetic field [15]. Although IMS has been used frequently for the recovery of specific microorganisms from different samples, the recovery rate of the microorganisms is rather low [8, 15, 17]. After separation of the cells from the sample several analysis methods such as plating, PCR and solid-phase cytometry (SPC) can be used to quantify the number of microorganisms.

In SPC, the principles of epifluorescence microscopy and flow cytometry are combined. Microorganisms are retained on a membrane filter, fluorescently labelled and automatically counted by the Chemscan RDI laser-scanning device. Subsequently, the data for each fluorescent spot are analysed by a computer to differentiate between fluorescent microorganisms and particles. Each retained spot can visually be inspected using an epifluorescence microscope [11, 20]. Due to its low detection limit, speed and the possible use of taxonomic probes for identification, SPC has the potential to overcome the shortcomings of other methods for quantification of *Candida* species in blood samples [7, 14].

In the present study, a method for the rapid quantification of *Candida* species and identification of *C. albicans* in whole blood based on IMS and SPC is described. This method was optimised using spiked blood samples and subsequently used to analyse 16 blood samples from high-risk patients.

MATERIALS AND METHODS

IMS/SPC method. 30 µl polyclonal anti-*C. albicans* antibody conjugated with fluorescein isothiocyanate (FITC) (Acris Antibodies, Herford, Germany) and 30 µl monoclonal anti-FITC antibody bound to microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to an EDTA-treated whole blood sample (max. volume: 15 ml). After incubation at room temperature for one hour with head-to-tail rotation, the sample was loaded on a whole blood column (Miltenyi Biotec). Prior to loading, the column was inserted into a QuadroMACS cell separator (Miltenyi Biotec) and prewashed with 3 ml of separation buffer (phosphate buffered saline (PBS), 0.5% bovine serum albumin and 2 mM EDTA, pH 7.2). Afterwards, the column was washed three times with 3 ml separation buffer, removed from the magnetic field and the yeasts were eluted with 5 ml elution buffer (Miltenyi Biotec).

The eluate was filtered over a 2.0 µm Cycloblack-coated polyester membrane filter (AES-Chemunex, Ivry-sur-Seine, France) and filters were incubated at 55°C for 30 min with 100 µl of PNA^{Flow} reagent (AdvanDx, Vedbaek, Denmark) containing a FITC-conjugated peptide nucleic acid (PNA) probe specific for *C. albicans* [19]. Subsequently, filters were incubated with 100 µl Wash Buffer^{Flow} (AdvanDx) for 10 min at 55°C. Finally, tyramide signal amplification was used to obtain red fluorescent *C. albicans* cells. To this end, the filter was placed on top of 100 µl polyclonal anti-FITC antibody conjugated with horseradish peroxidase (AbD Serotec, Oxford, UK) (10 µg/ml PBS) for 30 min at 30°C. A tyramide Alexa Fluor derivative (Invitrogen, Leek, the Netherlands) was then converted to its red fluorescent form by incubating the filter on 100 µl tyramide solution (stock solution diluted 1:50 in amplification buffer) for 30 min at 30°C. Between different steps, filters were rinsed three times with 1 ml PBS. Finally, all viable microorganisms were fluorescently labelled (green)

by incubating the filters at 37°C for one hour on a cellulose pad saturated with 600 µl ChemChrome V6 viability staining solution (stock solution diluted 1:100 in the labelling buffer ChemSol B2) (AES-Chemunex). With this procedure, all fungal cells retained on the filter are labelled green.

The filters with labelled microorganisms were placed on a holder, on top of a support pad (AES-Chemunex) moistened with 100 µl of Chemsol B2 (AES-Chemunex) and scanned by the ChemScan *RDI* (AES-Chemunex). This solid-phase cytometer consists of an argon laser, emitting light of 488 nm and two photomultiplier tubes, that detect the fluorescent light emitted by the labelled cells. The produced signals are processed by a PC, applying a series of software discriminants to differentiate valid signals associated with labelled *Candida* cells from other fluorescent particles. Subsequently, results are displayed as green spots on a membrane filter image in a primary and, after software elimination of background, secondary scan map [14]. To confirm the results, visual inspection using an Olympus BX40 epifluorescence microscope (Olympus, Tokyo, Japan) was performed. The filter holder was placed on a computer-driven moving stage in exactly the same position as in the Chemscan *RDI*. Subsequent highlighting of a green spot in the secondary scan map on the PC resulted in the direction of the microscope to the respective position on the membrane filter [2, 12], which allowed rapid visual inspection of all fluorescent spots. The red fluorescence of *C. albicans* cells can easily be observed using a 590 nm cutoff emission filter [20].

Preparation of the spiking solutions. In order to validate the IMS/SPC procedure, several experiments with spiked blood samples were carried out. An overview of the fungi used in these experiments is given in Table 1. These fungi were

cultured overnight at 30°C in Sabouraud liquid medium and serial dilutions were made in physiological saline. Subsequently, the number of cells was determined by SPC prior to spiking [20] and one ml of the obtained cell suspensions was used to spike the blood samples. For all spiking experiments, blood was freshly drawn from healthy volunteers, maintained at 4°C and used in experiments the same day.

Evaluation of the sensitivity and specificity of the assay. For sensitivity testing, the recovery of all *Candida* species (27 strains) was determined by analysis of spiked blood samples (10 to 50 *Candida* cells/10 ml of blood) with IMS/SPC. The specificity of the antibody for immunomagnetic capture of *Candida* cells was tested by spiking blood with various fungi not belonging to the genus *Candida* (11 strains). Additionally, the specificity of the PNA probe for identification of *C. albicans* was examined using all other *Candida* strains (16 strains) (Table 1).

Determination of the detection limit. In order to determine the detection limit of the IMS/SPC assay, a suspension of *C. albicans* ATCC MYA-2876 (76 cells/ml as determined by SPC) was serially diluted (6 times) and the number of cells present in these dilutions was also determined by SPC. Subsequently, blood samples from healthy volunteers were spiked with one ml of these solutions and the recovery with the IMS/SPC procedure was determined.

Comparison of IMS/SPC with blood culture (spiked blood). In order to compare the IMS/SPC method with conventional blood culture, five whole blood samples (10 ml) were obtained from ten healthy volunteers. One unspiked sample per healthy volunteer was used as a negative control and was analysed with the IMS/SPC

assay simultaneously with the other samples. Two samples per volunteer were spiked with *C. albicans* ATCC MYA-2876 and the other two were spiked with *C. dubliniensis* IHEM 14280, *C. glabrata* MUCL 29833, *C. krusei* IHEM 1795, *C. parapsilosis* IHEM 3270 or *C. tropicalis* MUCL 29952. The whole blood samples were spiked with 1 to 100 *Candida* cells. Two spiked whole blood samples were analysed immediately by IMS/SPC and two were analysed by conventional blood culture. To that end, the latter two spiked blood samples were aseptically introduced into a BacT/ALERT FA blood culture bottle (Biomérieux, Marcy l'Etoile, France) and the bottle was incubated at 37°C. The change of the color of the indicator was monitored visually.

Storage of blood samples. In order to investigate if storage of the blood samples at 4°C has an influence on the viability of the *Candida* cells, analysis was performed immediately and three, six, nine, 16 and 24 h after spiking. To that end, two times six blood samples (5 ml) from a healthy volunteer were spiked with 24 or 76 *C. albicans* ATCC MYA-2876 cells (cell number determined by SPC) and stored at 4°C until analysis.

Proof of concept by analysis of clinical samples. Finally, 16 samples from 15 patients with candidemia or suspected candidemia were analysed (Table 2). This research was approved by the ethical comitee of University Hospitals Leuven (Belgium) (study number S51459). Blood samples were stored at 4°C until analysis and all samples were analysed within 24 h of collection (Table 2). An equal aliquot of the blood sample was analysed both by the IMS/SPC procedure and by using a BacT/ALERT FA blood culture bottle that was monitored visually. If the blood

culture was positive, a subculture on Chromagar (BD) was made and subsequent identification of all yeasts recovered was performed using the API-20C AUX system (Biomérieux).

RESULTS

Evaluation of the sensitivity and specificity of the assay. The sensitivity of the assay was confirmed as for all *Candida* strains tested, the average recovery (± standard deviation) was 114% (± 20%) (n=27) after IMS/SPC analysis. The method proved highly specific and only cells belonging to *Candida* species were retained using the IMS procedure (Table 1). The specificity of the PNA probe was confirmed as only *C. albicans* cells yielded red fluorescence (Table 1).

Determination of the detection limit. A linear correlation between the number of cells (ranging from 0 to 76 as determined with SPC prior to spiking) in the spiking solutions and the number of cells found in the blood samples supplemented with this solution was obtained (data not shown). The average recovery (± standard deviation) was 116% (± 18%), and the detection limit was one cell per 10 ml of blood.

Comparison of IMS/SPC with blood culture (spiked blood). In order to compare the results obtained with IMS/SPC and conventional blood culture, we analysed spiked blood samples of ten healthy volunteers in parallel. The average recovery of *Candida* cells with the IMS/SPC procedure was 95% ± 29% (n=20) and was independent of the species. The absolute differences in number of cells in the spiking solutions and blood samples analysed by IMS/SPC were very small (Fig. 1). No *Candida* cells were detected in any of the negative control samples. A visual color change of the CO_2 sensor in all blood culture bottles was seen within 25 hours.

Storage of blood samples. We also determined whether storage (4°C) of blood samples had an influence on the number of yeast cells detected and could not observe

a reduction in the number of cells when the sample was analysed within nine hours of storage (Fig. 2). However, a marked decline (as high as 75%) in the number of cells, (independent of the absolute number) was found after 16 hours of storage (Fig. 2).

Proof of concept by analysis of clinical samples. Sixteen clinical samples were analysed with IMS/SPC and blood culture. Three samples were negative with both methods, six were positive with both methods and seven were positive with IMS/SPC but negative with blood culture. For some samples, visual and automated detection of a change in color of the indicator on the bottom of the blood culture bottle was compared and the same result was obtained (data not shown). Additionally, upon subculture, no *Candida* cells were isolated from any of the negative blood culture bottles.

Absolute counts obtained with IMS/SPC ranged from 2 to 480 *Candida* cells per ml (Table 2). For two patients, an extremely high number of *Candida* cells was found in the blood sample (> 175/ml for patient C and 480/ml for patient F). In both cases, a contaminated catheter was removed after collection of the sample. For patient C, a follow-up sample was obtained nine days after removal of the catheter and the start of anidulafungin treatment. This sample contained less cells (IMS/SPC analysis: 2 cells/ml) and was no longer positive with blood culture (Table 2).

The differentiation between *C. albicans* and other *Candida* species obtained with IMS/SPC was in agreement with the results obtained with culture for the six samples which were positive with both methods (Table 2). For the clinical samples negative with blood culture in this study and positive with IMS/SPC, the differentiation between *C. albicans* and other *Candida* species obtained with the IMS/SPC assay, was in agreement with the identity of the isolate recovered before

246 inclusion in the present study. Patients A, E, K, M and N presented with a mixed
247 infection according to the IMS/SPC analysis while this was not observed using the
248 culture-based method (Table 1). For patient I, *C. glabrata* and *C. albicans* were
249 isolated from a blood culture three days before inclusion in our study. With IMS/SPC
250 only *C. albicans* cells were found in the sample.
251

DISCUSSION

We report the development of a four-hour method for the quantification of *Candida* species in blood, based on IMS and SPC using viability staining and fluorescent in situ hybridisation (FISH) to label all *Candida* species and *C. albicans*, respectively. Analysis of spiked blood samples indicated that our method is specific, has a low detection limit and allows accurate quantification.

Using spiked blood samples we did not observe an improved diagnostic sensitivity with the IMS/SPC method. Unlike in numerous reports in literature, all blood cultures of spiked samples were positive [4, 10, 18]. This can be explained by the use of laboratory strains to spike the blood cultures. However, seven clinical samples were positive with IMS/SPC but negative with blood culture. The latter observation clearly indicates that the IMS/SPC assay has a higher diagnostic sensitivity than blood culture. For four of the samples for which blood culture was negative but IMS/SPC was positive, treatment with antifungal drugs prior to collection might be an explanation for the divergent results (Table 2).

We observed that analysis of blood samples within nine hours of sample collection is recommended due to a reduction in the number of yeast cells recovered, after longer storage. Nevertheless, even for samples that were analysed more than 9 h past sampling (Table 2), we were able to quantify the *Candida* species cells present, although it is likely that the number observed is an underestimation of the number of cells actually present at the time of sampling.

The IMS/SPC method does not only allow the rapid diagnosis of candidemia but also the accurate quantification of *Candida* cells. This might help in assessing the response to antifungal therapy or may lead to the decision to remove intravascular devices [18]. Our data suggest that a high number of *Candida* cells present in a blood

277 sample is associated with a contaminated intravascular device [18], although only two
278 samples were investigated and further research is required to support this hypothesis.
279 The present study also demonstrates that mixed infections might occur more
280 frequently than generally assumed from culture-based techniques. While in five of the
281 16 clinical samples *C. albicans* and at least one other *Candida* species was found
282 using the IMS/SPC procedure, not a single mixed infection was identified using blood
283 culture.
284
285 Only one previous study has used IMS for the recovery of *Candida* cells from
286 spiked blood [1]. However, this study used culture after capture of the cells and
287 therefore results were only obtained one day before results could normally be
288 obtained with blood culture. Additionally, identification of the *Candida* species was
289 only possible after culture and some species were not captured by the antibody.
290 Finally, the low recovery (11 to 43%) observed for spiked blood samples was a
291 crucial problem limiting the use of the method for the analysis of clinical samples
292 containing low numbers of *Candida* cells [1].
293 Several research groups have obtained good results when using PCR on DNA
294 isolated from blood for the diagnosis of candidemia [5, 13, 22]. The time to result
295 using a commercial real-time PCR assay (Septi*Fast* by Roche) is six hours which is
296 comparable with our method. However, one of the problems of PCR methods is the
297 possible detection of DNA from dead and/or degrading cells instead of living yeasts.
298 To avoid this, a labelling step using the viability stain Chemchrome V6 was included
299 in the SPC procedure described in the present study.
300 PNA FISH methods are increasingly being used in hospitals for the
301 identification of *Candida* species after the detection of yeasts in blood culture bottles.

In our study, we used a commercially available PNA probe for *C. albicans* identification but by using IMS and SPC we could omit the time-consuming culture step [19]. The implementation of additional PNA probes allowing the identification of other *Candida* species might further improve our method.

In conclusion, our study provides a proof of principle for the diagnostic potential of IMS combined with SPC for the detection of *Candida* cells in whole blood. In its current form, the method may be too laborious for implementation in a routine diagnostic laboratory. Further automation of the technique combined with an expansion of the panel of pathogens that can be detected and identified in blood could make this technique more attractive for diagnostic laboratories in the future.

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403 **Table 1:** Overview of the fungi used for sensitivity and specificity testing and their

404 reactivity with the capturing antibody and the PNA probe used in the assay.

Species	Strain designation [a]	Reactivity with the antibody used for immunomagnetic capture	Reactivity with the PNA probe used for identification of *C. albicans*
Candida albicans	ATCC MYA-682	+	+
	ATCC MYA-2876	+	+
	ATCC 10231	+	+
	IHEM 9559	+	+
	IHEM 10284	+	+
	MUCL 29903	+	+
	MUCL 29800	+	+
	MUCL 29919	+	+
	MUCL 29981	+	+
	MUCL 30112	+	+
	NCYC 1467	+	+
Candida dubliniensis	IHEM 14280	+	-
Candida famata	CI	+	-
Candida glabrata	MUCL 15664	+	-
	MUCL 29833	+	-
Candida guilliermondii	CI	+	-
Candida inconspicua	CI	+	-
Candida kefyr	CI	+	-
Candida krusei	IHEM 1796	+	-
Candida lusitaniae	CI	+	-
Candida maris	CI	+	-
Candida parapsilosis	IHEM 3270	+	-
Candida pseudotropicalis	CI	+	-
Candida tropicalis	IHEM 4222	+	-
	IHEM 4225	+	-
	MUCL 29952	+	-
	MUCL 30002	+	-
Aspergillus flavus	IHEM 2700	-	-
Aspergillus fumigatus	IHEM 3768	-	-
Cryptococcus neoformans	IHEM 14513	-	-
Fusarium graminearum	IHEM 2994	-	-

Fusarium oxysporum	IHEM 14513	-	-
Fusarium solani	IHEM 6743	-	-
Penicillium chrysogenum	IHEM 3035	-	-
Penicillium simplicissimum	IHEM 1202	-	-
Pseudallescheria boydii	CBS 101.22	-	-
Saccharmyces cerevisiae	ATCC 9763	-	-
Scedosporium prolificans	CBS 467.74	-	-

405 [a] ATCC: American Type Culture Collection; CBS: Centraalbureau voor
406 Schimmelcultures (the Netherlands); CI: clinical isolate from our own collection;
407 IHEM: Instituut voor Hygiene en Epidemiologie (Belgium); MUCL: Mycothèque de
408 l' Université Catholique de Louvain (Belgium); NCYC: National Collection of Yeast
409 Cultures (UK).

410

411 **Table 2:** Overview of the characteristics of the clinical samples included in the study and the results obtained after analysis with a traditional

412 blood culture and with the method combining immunomagnetic separation and solid-phase cytometry (IMS/SPC).

Patient	Blood culture result obtained before inclusion in this study	Sample collection date	Analysis date	Treatment prior to sample collection	Results of the analysis of the clinical samples		
					Blood culture	Number of cells/ml of blood (IMS/SPC)	
						C. albicans	other *Candida* spp.
A	-[a]	06-16-09	06-16-09	-[b]	negative	3	3
B	*C. glabrata*	08-10-09	08-11-09	Fluconazole	negative	0	2
C	*C. tropicalis*	08-11-09	08-12-09	Fluconazole	*C. tropicalis*	0	> 175[c]
C	-[a]	08-20-09	08-20-09	Fluconazole & anidulafungin	negative	0	2
D	*C. albicans*	08-20-09	08-20-09	Fluconazole	negative	3	0
E	*C. glabrata*	08-20-09	08-21-09	-[b]	*C. glabrata*	3	4
F	*C. glabrata*	08-20-09	08-21-09	Fluconazole & caspofungin	*C. glabrata*	0	480
G	*C. albicans*	09-14-09	09-15-09	Fluconazole	negative	0	0
H	*C. tropicalis*	09-14-09	09-15-09	Fluconazole	negative	0	0
I	*C. albicans*	09-14-09	09-15-09	Fluconazole	*C. albicans*	2	0

	C. glabrata						
J	*C. glabrata*	10-06-09	10-07-09	-[b]	*C. glabrata*	0	4
K	*C. glabrata*	10-07-09	10-08-09	-[b]	negative	6	3
L	-[a]	10-07-09	10-08-09	-[b]	negative	0	0
M	*C. parapsilosis*	10-08-09	10-09-09	Fluconazole	negative	1	6
N	*C. albicans*	12-04-09	12-05-09	Fluconazole	*C. albicans*	1	1
O	*C. krusei*	01-04-10	01-05-10	Caspofungin	negative		

413 [a] No positive blood culture was obtained prior to inclusion in this study.

414 [b] Patients did not receive treatment prior to inclusion in this study.

415 [c] Of this blood sample 4 ml was filtered and more than 700 *Candida* cells were present on the filter but a reliable quantification was impossible.

416 Filtration of a smaller volume could have led to a more reliable quantification.

417

Figure legends:

Fig. 1: Numbers of *Candida* cells in the spiking solution (white bars) compared to the numbers obtained after application of the IMS/SPC procedure on spiked blood samples of ten healthy volunteers (grey bars).

Fig. 2: The number of *C. albicans* cells obtained with the IMS/SPC procedure for two spiked blood samples from one healthy volunteer (triangles and squares respectively) determined at different time-points during storage of the samples at 4°C.